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# Coexistence of Two Distinct Copies of Naphthalene Degradation Genes in *Pseudomonas* Strains Isolated from the Western Mediterranean Region

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**We analyzed the occurrence of the naphthalene degradation upper-pathway (*nah*) genes in the western Mediterranean region. The amplification, restriction, and sequence analysis of internal fragments for several *nah* genes (*nahAc*, *nahB*, *nahC*, and *nahE*) from naphthalene-degrading strains isolated from this geographical area proved the coexistence of two distinct sets of *nah* genes.**

Naphthalene degradation has been extensively studied in several *Pseudomonas* strains, such as the archetypes *Pseudomonas putida* G7 (11, 23, 25, 29) and *P. putida* NCIB9816 (5, 19). In both strains, the dissimilatory genes are organized into two operons: one coding for the enzymes involved in the conversion of naphthalene to salicylate (*nahAaAbAcAdBFCED*, naphthalene degradation upper pathway) and the second coding for the conversion of salicylate to tricarboxylic acid cycle intermediates (pyruvate and acetyl coenzyme A) through the meta-cleavage pathway (*nahGTHINLOMKJ*, naphthalene degradation lower pathway). Regulation of both operons is mediated by a single protein, NahR, that acts as a positive regulator for both promoters, with salicylate being the inducer of the system (23).

Nucleotide sequences coding for the entire naphthalene degradation upper pathway have been determined in several *Pseudomonas* species such as *Pseudomonas* sp. strain C18 (6), *P. putida* OUS82 (27), *Ralstonia* sp. strain U2 (7), *P. stutzeri* AN10 (4), *P. putida* BS202 (GenBank accession no. AF010471), and *P. aeruginosa* PaK1 (GenBank accession no. D84146). Interestingly, comparison of this last sequence with the sequences for *P. stutzeri* AN10 and for *Pseudomonas* sp. strain C18 revealed that the upper pathway of strain PaK1 could be the result of a recombination event between the upper pathways found in the other two strains (4). In this manner, it has been suggested that a *P. aeruginosa* PaK1 ancestor recruited two entire naphthalene degradation upper pathways and a mosaic upper pathway was selected for, probably due to environmental pressure, in the ancestral strain of PaK1 (4). Other mosaic patterns within individual degradative genes or within catabolic operons as a result of recombination between homologous sequences have been previously reported (1, 2, 10), and the same is true for the coexistence of nearly identical metabolic modules (i.e., pWW53 and related plasmids) (24, 28).

Our objectives in the present study were (i) to analyze the presence of two distinct sets of *nah* upper-pathway genes in the

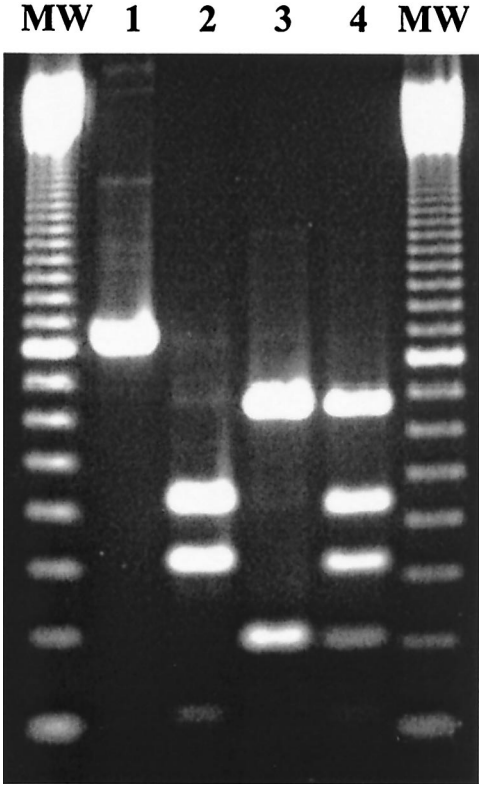


FIG. 1. Agarose gel showing the *Hae*III restriction products of the intra-*nahAc* amplicon. Lanes: 1, nondigested 866-bp intra-*nahAc* PCR product from *P. stutzeri* AN10; 2, digested PCR product from *Pseudomonas* sp. strain C18; 3, digested PCR product from *P. stutzeri* strain AN10; 4, digested PCR product from *Pseudomonas* sp. strain $5_{III}$ASal. MW, 100-bp molecular mass marker (Gibco-BRL).

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TABLE 1. Naphthalene degradation strains used for this study

| Presence (+) or absence (−) of key physiologic markers[a] | | | | *nahAc* type[b] | Strain(s)[c] | Origin, date of isolation (yr)[d] | Source or reference |
|---|---|---|---|---|---|---|---|
| GEL | STA | NAR | ARG | | | | |
| − | + | + | − | AN10 | **AN10, AN11** | Marine isolate, Barcelona, 1982 | 9 |
| | | | | | **LSMN2, ST27MN2, ST27MN3, LS401** | Marine isolate, Barcelona, 1988 | 21 |
| | | | | | **SP1402** | Wastewater, Mallorca, 1988 | 21 |
| | | | | | B1SMN1, **B2SMN**, S1MN1 | Wastewater, Menorca, 1988 | 21 |
| − | + | + | + | AN10 | **LSMN7**, ST27MN1, ST27401, ST27402, ST27403, 19SDN3, 19SMN5, 19SMN6 | Marine isolate, Barcelona, 1988 | 20 |
| + | + | + | + | AN10 | **LSMN3** | Marine isolate, Barcelona, 1988 | 20 |
| − | − | + | − | AN10 | **SP401** | Wastewater, Mallorca, 1988 | 20 |
| | | | | | 19SDN2 | Marine isolate, Barcelona, 1988 | 20 |
| + | − | + | + | AN10 | 5NH(5a), $5_{II}$DSal, 9NH, 16NH, $16_{I}$DSal, $23_{III}$ASal | Marine isolate, Barcelona, 1987 | 8 |
| | | | | | **LS402** | Marine isolate, Barcelona, 1988 | 20 |
| | | | | | SP1403 | Wastewater, Mallorca, 1988 | 20 |
| + | − | + | + | C18 | **$8_{I}D_{I}$NH**, $14_{I}$DNH, $18_{I}$D2NH, $32_{II}$NH | Marine isolate, Barcelona, 1987 | 8 |
| − | − | − | − | AN10 | **S1MN3** | Wastewater, Menorca, 1988 | 20 |
| − | − | − | + | AN10 | **$3_{III}$A2NH**, $4_{I}$ASal, $6_{II}$ANH, $11_{III}$DNH | Marine isolate, Barcelona, 1987 | 8 |
| | | | | | LSMN6 | Marine isolate, Barcelona, 1988 | 20 |
| − | − | − | + | C18 | $1_{II}$A2NH, **$3_{I}$A2NH**, $8_{I}$DNH, $15_{I}D_{I}$NH, $15_{I}$D2NH, $15_{III}$D, $17_{II}$DNH, $19_{II}$DNH, $23_{I}$DNH, $24_{I}$NH, $26_{I}$NH, $27_{I}$NH, $28_{I}$2NH, $30_{I}$NH, $33_{II}$NH, 34JB, $34_{I}$NH, $34_{II}$NH, | Marine isolate, Barcelona, 1987 | 8 |
| | | | | | **PR1MN1**, PR2MN1, PR3MN2 | Wastewater, Mallorca, 1988 | 20 |
| − | − | − | + | AN10 + C18 | **$2_{I}D_{I}$NH, $5_{III}$ASal**, $7_{II}$ANH, $10_{I}D_{I}$NH, 11NH | Marine isolate, Barcelona, 1987 | 8 |
| | | | | | S1MN2 | Wastewater, Menorca, 1988 | 20 |

[a] All strains were identified as members of the genus *Pseudomonas*, being catalase-positive, motile, gram-negative, rod-shaped bacteria with a strict aerobic respiratory metabolism (18). Supplementary key physiologic markers were as follows: GEL, hydrolysis of gelatin; STA, hydrolysis of starch; NAR, denitrification capability; and ARG, presence of arginine hydrolase.

[b] Classification was performed according to the *Hae*III restriction pattern observed in Fig. 2.

[c] Strains in boldface were selected as representative strains and were used for 16S rRNA and *nahAc*-like phylogenetic studies. Single-underlined strains had been previously identified as *P. stutzeri* (21, 22). Double-underlined strains had been previously identified as *P. balearica* (3).

[d] That is, Barcelona, Spain, Mallorca, Spain, and Menorca, Spain.

western Mediterranean region and (ii) to prove that these two distinct types of genes could physically coexist in the same host strain.

**Amplification of *nahAc* homologues from naphthalene-degrading *Pseudomonas* strains.** Naphthalene dioxygenase (NahA) catalyzes the first step in the degradation of naphthalene, the *cis*-dihydroxylation reaction (12), being a three-component class III oxygenase (ferredoxin [NahAb], ferredoxin reductase [NahAa], and terminal dioxygenase [NahAcAd, also NDO]) in which terminal dioxygenase is an $\alpha_3\beta_3$ hexamer (13). Lloyd-Jones and coworkers (14, 15) affiliated the NDO large subunit ($\alpha$, NahAc-like) gene sequences with three major branches: the *nah*-like group, the *dnt*/*ntd* group, and the *phn*-type group.

Sequences for NDO large subunit genes from *Rhodococcus* sp. strain NCIMB12038 (*narAa* [GenBank accession no. AF082663]), *Burkholderia* sp. strain RP007 (*phnAc* [AF061751]), *Alcaligenes faecalis* AFK2 (*phnAc* [AB024945]), *Ralstonia* sp. strain U2 (*nagAc* [AF036940]), *Comamonas testosteroni* H (*pahAc* [AF252550]), *Burkholderia* sp. strain DNT (*dntAc* [U62430]), *Pseudomonas* sp. strain JS42 (*ntdAc* [U49504]), *Pseudomonas stutzeri* AN10 (*nahAc* [AF039533]), *P. aeruginosa* PAK1 (*pahA3* [D84146]), *P. putida* G7 (*nahAc* [M83949]), *P. fluorescens* ATCC 17483 (*ndoC2* [AF004283]), *P. putida* OUS82 (*pahAc* [AB004059]), *P. putida* ATCC 17484 (*ndoC2* [AF004284]), *P. putida* NCIB9816 (*ndoB* [M23914]), *P. putida* NCIB9816–4 (*nahAc* [M83950]), *P. putida* BS202 (*nahA3* [AF010471]), and *Pseudomonas* sp. strain C18 (*doxB* [M60405]) were aligned and forward (Ac149f, 5′-CCCYGGC GACTATGT-3′) and reverse (Ac1014r, 5′-CTCRGGCATG TCTTTTTC-3′) degenerated primers were chosen in conserved regions among the genes belonging to the *nah*-like and *dnt*/*ntd* groups. In this regard, it was not possible to target the less related *phn*-type dioxygenases.

Primers were used on 66 naphthalene-degrading *Pseudomonas* strains isolated from the western Mediterranean region. These strains had been isolated in our laboratory over the last 20 years and were able to grow with naphthalene as the unique energy and carbon source (Table 1). A single PCR fragment of the predicted size (866 bp) was amplified in all of them (Fig. 1, lane 1). In order to evaluate the genetic diversity of the naphthalene dioxygenase genes, PCR fragments were digested with the restriction enzyme *Hae*III. These strains clustered in three groups. The first group consisted of a *nah*-like AN10 group, with a restriction pattern similar to *nahAc* from *P. stutzeri*

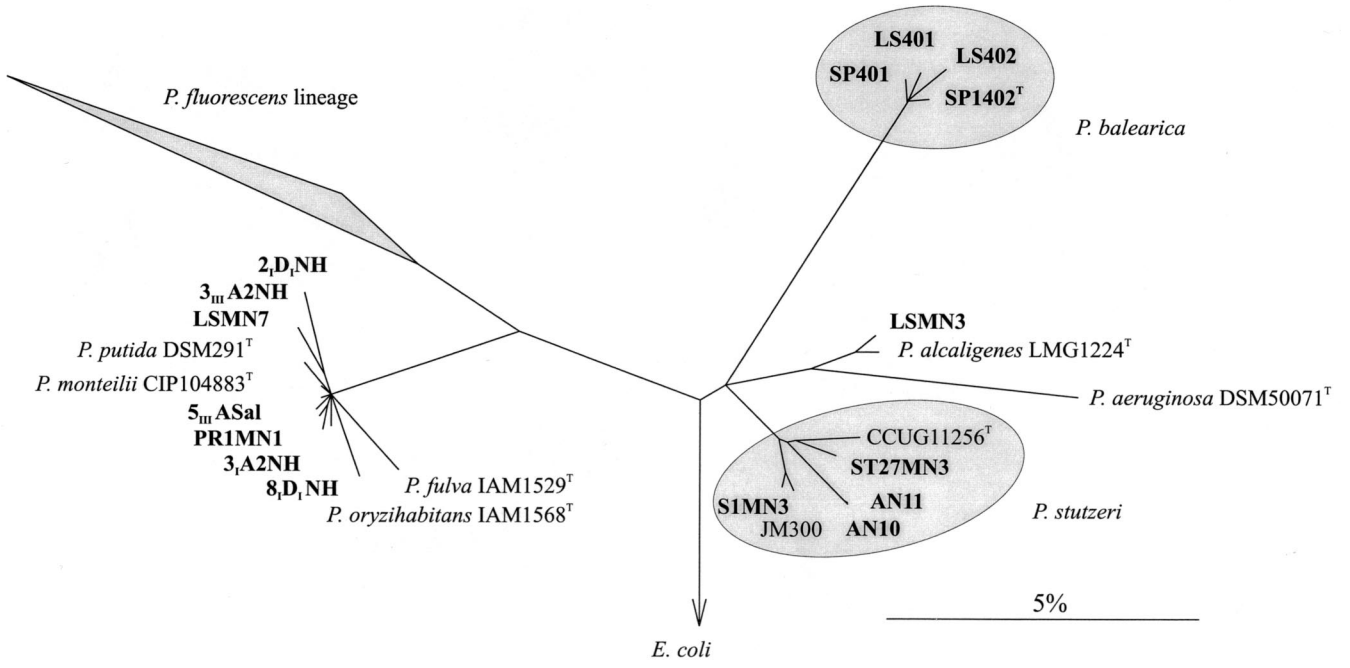


FIG. 2. 16S rRNA-based tree reflecting the phylogenetic affiliation of the sequenced strains. The tree is based on the results of a parsimony analysis that included only complete or nearly complete 16S rRNA sequences of representative bacteria (17). The topology of the tree was corrected as described by Ludwig et al. (16). Multifurcations indicate where a topology could not be resolved unambiguously. The phylogenetic positions of partial sequences resulted from the insertion of the aligned sequences into the tree by using the parsimony ARB tool (26) without modifying its topology during the sequence positioning. Group names indicate the most representative organism of the phylogenetic branch. The bar indicates 5% estimated sequence divergence. Representative sequences shown in the tree and their accession numbers were as follows: *P. putida* DSM291$^T$ (Z76667), *P. monteilii* CIP104883$^T$ (AB021409), *P. fulva* IAM1529$^T$ (D84015), *P. oryzihabitans* IAM1568$^T$ (D84004), *P. alcaligenes* LMG1224$^T$ (Z76653), *P. aeruginosa* DSM50071 (X06684), *P. balearica* SP1402$^T$ (U26418), *P. balearica* LS401 (U26417), *P. stutzeri* CCUG11256$^T$ (U26262), *P. stutzeri* ST27MN3 (U26419), *P. stutzeri* AN11 (U25280), *P. stutzeri* AN10 (U22427), and *P. stutzeri* JM300 (X98607). The sequences were submitted to EMBL as follows (with the accession numbers in parentheses): *Pseudomonas* sp. strain $2_tD_1$NH (AF307868), *Pseudomonas* sp. strain $3_t$A2NH (AF307866), *Pseudomonas* sp. strain $3_{III}$A2NH (AF307865), *Pseudomonas* sp. strain LSMN7 (AF307871), *Pseudomonas* sp. strain $8_tD_1$NH (AF307872), *Pseudomonas* sp. strain $5_{III}$ASal (AF307864), *Pseudomonas* sp. strain PR1MN1 (AF307867), *P. alcaligenes* LSMN3 (AF307870), *P. stutzeri* S1MN3 (AF307873), *P. balearica* LS402 (AF307874), and *P. balearica* SP401 (AF307875).

AN10 (4) and *pahA3* from *P. aeruginosa* PaK1 (Fig. 1, line 3) (35 strains, 53% of the total). The second group was the *nah*-like C18 group, with a pattern similar to *doxB* from *Pseudomonas* sp. strain C18 (6) and *nahAc* from *P. putida* G7 (25) (Fig. 1, line 2) (25 strains, 38% of the total). The third group was composed of six strains (9% of the total) with identical restriction patterns (Fig. 1, lane 4), suggesting by the sum of the resulting fragments the coexistence of both AN10 and C18 naphthalene dioxygenase genes in each of these strains.

**Grouping and selection of representative strains.** In order to select a reduced number of strains to be further studied, strains were grouped according to several key physiological characteristics that are discriminative among true pseudomonads (18). The results were compared with the *nahAc* restriction pattern type, and a tentative strain grouping was achieved (Table 1). One or two representatives in each group were selected, and their 16S rRNA was sequenced (indicated in boldface in Table 1). Almost complete sequences of strains $5_{III}$ASal, $3_{III}A_2$NH, $3_IA_2$NH, PR1MN1, and $2_ID_1$NH were aligned and their phylogeny reconstructed by using the current database of ca. 20.000 aligned sequences (17). Once a consensus tree was drawn (Fig. 2), aligned partial sequences of strains LSMN3, LSMN7, $8_ID_I$NH, S1MN3, LS402, and SP401 were added to this tree by using the parsimony tool of the ARB program (26) to determine their affiliation. As shown in Fig. 2, new sequences were affiliated with four different branches within the true members of the genus *Pseudomonas*. The phylogenetic affiliation did not match with the groups made after physiological similarities, showing a relatively poor value of the key characteristics chosen. Most of them (7 of 11) were affiliated with the *P. putida-P. monteilii* branch; thus, we could not assign them to any of the known species because of the extremely close relationships among the sequences of the members of this branch. One sequence, that of LSMN3, could be affiliated with *P. alcaligenes*. One sequence, that of S1MN3, could be affiliated with genomovar 8 of *P. stutzeri*, and two sequences, those of SP401 and LS402, could be affiliated with *P. balearica*.

***nahAc* gene fragment analysis.** The internal *nahAc* gene fragments from all selected strains (Table 1) were cloned and sequenced. The nucleotide sequences were aligned, and their phylogenies were reconstructed by using the neighbor-joining algorithm (Fig. 3). All *nahAc*-like sequences were affiliated with both AN10 and C18 branches. This confirmed the previously observed clustering observed by amplification and restriction analysis.

Upon comparing reconstructed phylogenies of the 16S rRNA gene and *nahAc* (Fig. 2 and 3, respectively) we found that (i) marine bacteria with different affiliations (i.e., *P.*

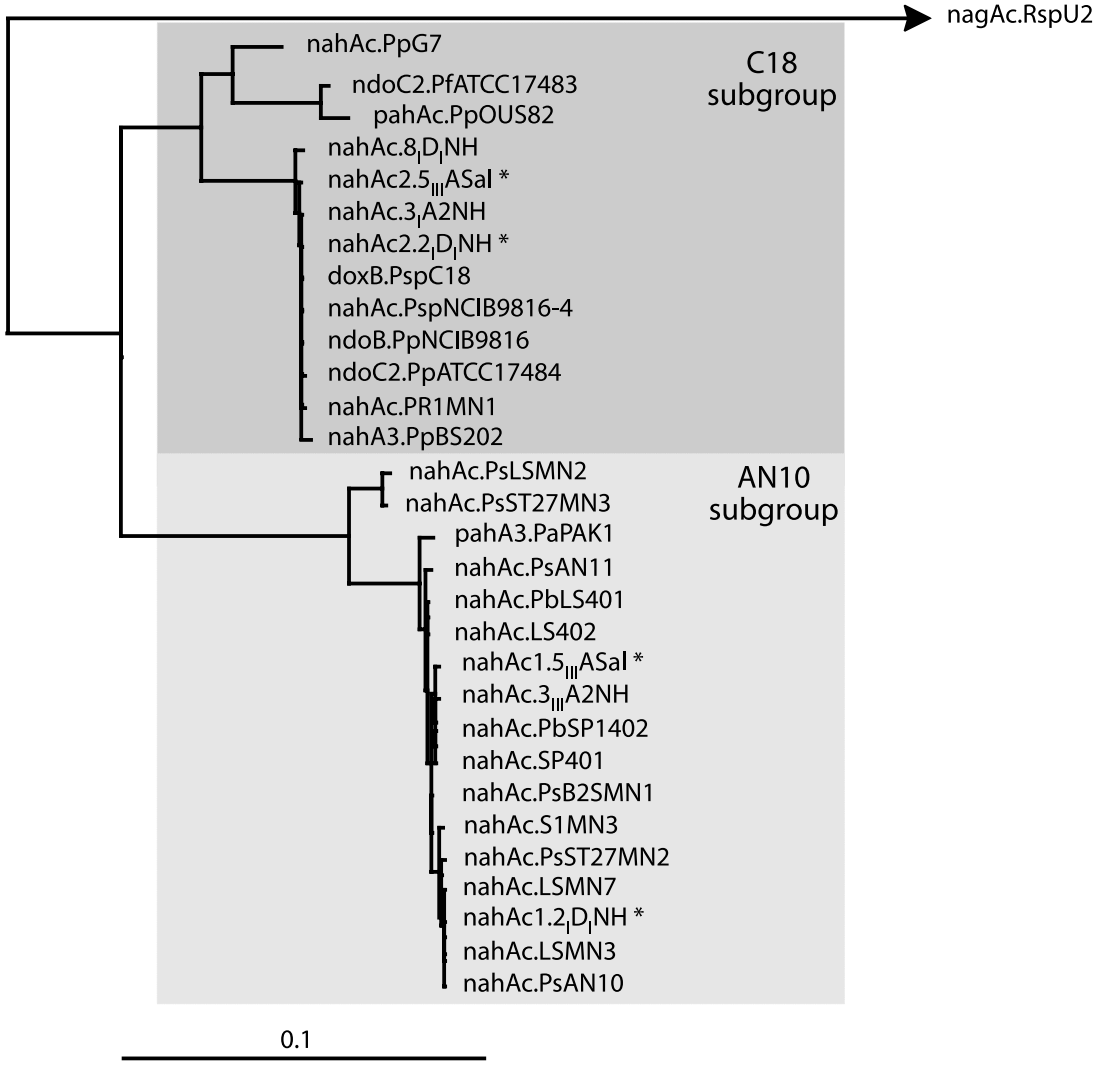


FIG. 3. Phylogenetic relationships between the intra-*nahAc* fragments obtained from the selected naphthalene-degrading strains and from strains retrieved from databases. The relative evolutionary distances were determined by using the program DNADist from the PHYLIPS package program. The tree has been drawn by using the program DrawTree from the same package based on pairwise similarity scores. The bar indicates 10% estimated sequence divergence. The name and GenBank-EMBL accession number of genes noted in plain characters are described in the text. The naphthalene-degrading strains analyzed in this study are noted in boldface type, with their names and *nahAc*-like gene GenBank-EMBL accession numbers (in parentheses) as follows: *Pseudomonas* sp. strain $8_{I}D_{I}NH$ (AF306433), *Pseudomonas* sp. strain $3_{I}A2NH$ (AF306436), *Pseudomonas* sp. strain PR1MN1 (AF306432), *P. stutzeri* LSMN2 (AF306423), *P. stutzeri* ST27MN3 (AF306425), *P. stutzeri* AN11 (AF306419), *P. balearica* LS401 (AF306420), *P. balearica* LS402 (AF306429), *Pseudomonas* sp. strain $3_{III}A2NH$ (AF306430), *P. balearica* SP1402$^{T}$ (AF306421), *P. balearica* SP401 (AF306428), *P. stutzeri* B2SMN1 (AF306424), *P. stutzeri* S1MN3 (AF306435), *P. stutzeri* ST27MN2 (AF306422), *Pseudomonas* sp. LSMN7 (AF306431), *P. alcaligenes* LSMN3 (AF306427), *Pseudomonas* sp. strain $2_{I}D_{I}NH$ (AF306437 [*nahAc1*] and AF306438 [*nahAc2*]), *Pseudomonas* sp. strain $5_{III}ASal$ (AF306439 [*nahAc1*] and AF306440 [*nahAc2*]). Asterisks denote strains containing two *nahAc* alleles.

*stutzeri* ST27MN2, *P. alcaligenes* LSMN3, *Pseudomonas* sp. strain LSMN7, and *P. balearica* LS401) but isolated from the same geographical location and at the same time (i.e., Barcelona, Spain, in 1988) harbored nearly identical *nahAc* genes (AN10 type) and (ii) marine bacteria with a common origin (Barcelona, Spain, in 1987) such as those affiliated with the *P. putida-P. monteilii* branch may harbor one of the two *nahAc* type genes (strain $3_{III}A2NH$, AN10 type, and strain $8_{I}D_{I}NH$, C18 type). Additionally, all strains with the complex *nahAc* restriction pattern (Fig. 1, lane 4) were confirmed to harbor both types of genes. Thus, it can be concluded that in the western Mediterranean region at least two distinct naphthalene dioxygenase genes can be found and may coexist within the same host strain. This might also be true for the rest of the naphthalene degradation upper-pathway genes.

**Coexistence of other *nah* upper-pathway genes.** In order to study the coexistence of two copies of other *nah* upper-pathway genes, sequences for the *nahB*-, *nahC*-, and *nahE*-like genes from *Pseudomonas* sp. strain C18 (*doxE*, *doxG*, and *doxI* [GenBank accession number M60405]), *P. stutzeri* AN10 (*nahB*, *nahC*, and *nahE* [AF039533]), *P. putida* OUS82 (*pahB*, *pahC*, and *pahE* [AB004059]), *P. putida* G7 (*nahB*, *nahC*, and *nahE* [AF125184, J04994, and U09057, respectively]), *P. aeruginosa* PaK1 (*pahB*, *pahC*, and *pahE* [D84146]), and *P. putida* BS202

(*nahB*, *nahC*, and *nahE* [AF010471]) were aligned, and amplification primers were designed for the conserved regions. The primers and expected PCR products were as follows: B6f (5′-CAATCAACAAGTCGTTTC-3′) and B778r (5′-ACTTGCGACCGAGCG-3′) were used to amplify an internal *nahB*-like fragment of 773 bp, C118f (5′-GAGAAGGACCGTTTCTATC-3′) and C814r (5′-CACCTCGCCAGCCGGG-3′) were used to amplify an internal *nahC*-like fragment of 697 bp, and E207f (5′-CGCYACGTTGACCTGGG-3′) and E826r (5′-CCGAAAAGTCGCCACGC-3′) were used to amplify an internal *nahE*-like fragment of 620 bp. Degeneracy in primer E207f was needed to accommodate all *nahE*-like genes. The primers were used on all strains harboring two distinct *nahAc*-like genes. A single PCR product, equal in size to the predicted amplicons, was amplified in all of them. The PCR products were restricted with *Taq*I, *Alu*I, or *Hae*III endonucleases and, in all cases, their restriction pattern suggested the presence of two distinct copies of each *nahB*, *nahC*, and *nahE* gene (results not shown). *Pseudomonas* sp. strain $5_{III}$ASal was selected to further clone and sequence the internal *nahB*-, *nahC*-, and *nahE*-like fragments. Sequence alignments confirmed the presence of two plausible functional copies for all genes. One of the alleles for each gene (*nahB1*, *nahC1*, and *nahE1*) was closer in sequence to its *nah* homologous gene present in *P. stutzeri* AN10 (4), while the other alleles (*nahB2*, *nahC2*, and *nahE2*) showed greater identity to their relative in the *dox* pathway of *Pseudomonas* sp. strain C18 (6) (*nahB1* of *Pseudomonas* sp. strain $5_{III}$ASal [AF320638], 99.2% identity to *nahB*, 86.9% identity to *doxE*; *nahB2* [AF320639], 89.9% identity to *nahB*, 96.9% identity to *doxE*; *nahC1* [AF320640], 98.5% identity to *nahC*, 85.5% identity to *doxG*; *nahC2* [AF320641], 85.7% identity to *nahC*, 99.7% identity to *doxG*; *nahE1* [AF320642], 95.9% identity to *nahE*, 94.8% identity to *doxI*; *nahE2* [AF320643], 90.4% identity to *nahE*, 100.0% identity to *doxI*). Thus, strains harboring two distinct *nahAc*-like genes may also have two copies of all naphthalene-degradation upper pathway genes: one homologous to the *nah* genes from *P. stutzeri* AN10 and another homologous to the ones present in *Pseudomonas* sp. strain C18.

In summary, our results reveal that closely related naphthalene-degrading bacteria (*Pseudomonas* spp.) isolated from the western Mediterranean region may independently harbor two distinct *nahAc*-type genes: the AN10 and the C18 types (Fig. 3). Furthermore, we have shown that, in some cases, both naphthalene dioxygenase-encoding genes and other *nah* upper pathway genes coexist in the same host strain. Since assimilation of polyaromatic hydrocarbons such as naphthalene and its derivatives is due to the combined action of several enzymes and not only to the action of a unique enzyme, the initial dioxygenase, plausible recombination events between homologous but distinct gene copies could result in new hybrid alleles of these genes. The corresponding gene products might show small differences in their amino acid sequence that could improve the degradation of naphthalene (and its derivatives), thereby conferring a selective advantage on these strains. In this sense, strains harboring these two nearly identical but distinct copies of the *nah* upper-pathway genes, such as *Pseudomonas* sp. strain $5_{III}$ASal and *Pseudomonas* sp. strain $2_ID_INH$, can be excellent models for studying the role of natural recombination between homologous catabolic pathways in accelerating their evolution and improving their biochemical capabilities. Thus, experiments to improve naphthalene degradation capabilities and analysis of their resulting *nah* upper-pathway gene combinations are called for.

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